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Variability in bacteriostatic properties of the larvae of *Hypoderma bovis*

Zmienność właściwości bakteriostatycznych larw *Hypoderma bovis*

The bacteriostatic substances of *Hypoderma bovis* larvae which were confirmed by Jettmar 1953 occurred in the entire intestine (from gullet till anus) in larvae pressed out from subcutaneous knobs i. e. at the third developmental stage according to the terminology of Knipling 1935.

Jettmar did not study *Hypoderma bovis* at more early developmental stages. He utilized the following species of bacteria for his study of bacteriostatic properties of the larvae investigated: *Sarcina rosa, Streptococcus scarlatinae,* several forms of *Staphylococci, Corynebacterium diphteriae, Bac. anthracis* spores, Gram-positive *Coccobacilli* from the guinea-pig and Gram-negative ones: *Eberthella typhosa, Salmonella paratyphi B, Salmonella dys. Flexner, Prodigiosus, Fluorescens liquefaciens, Pseudomonas aeruginosa, Serratia marcescens, Chromabact. violaceum* as well as *Proteus OX*-19 and *Proteus vulgaris.*

On the grounds of the results obtained, Jettmar carried out a comparison of the bacteriostatic properties of the *Hypoderma bovis* larvae confirmed by himself with those of visceral liquid of adult *Ascaris lumbricoides, Ascaris suum, Parascaris equorum* (Jettmar 1952, Pavan 1952), and of cuticular extracts of the above helminths (Jettmar 1952). In all of these cases, the Gram-positive bacteria were inhibited while the Gram-negative ones did not at all react in response to the effect of these substances. The used species of *Sarcina* proved the most sensitive one.

It results from the work by Stefański et Przyjałkowski 1959 that the bacteriostatic properties are absent at younger developmental stages in the *Nematoda,* namely: in the migratory larvae of *Ascaris suum, Strongylinae* and other *Nematoda* parasitizing domestic animals, although they occur in these *Nematoda* at the grown-up stage. In any case, bacteriostatic properties were confirmed in grown-up *Ascaris lumbricoides, Ascaris suum* and *Parascaris equorum.*

The question thus arises whether the bacteriostatic properties are present in *Hypoderma bovis* larvae at the younger developmental stages and if so, to what extent?

Material and methods

Hypoderma bovis larvae were taken from the canalis vertebralis beneath the mater dura cover. The definition of these larvae given by Gebauer 1952 seems correct: „Wanderlarve der Grossen Dasselfliege". This definition contains some analogy to the migratory larvae of the *Nematoda.*

The migratory larvae of *Hypoderma bovis,* seventy specimens of which were utilized in the present study, were obtained from cattle slaughtered in the City slaughter-house, Warszawa, during the period from February to April 21, 1960. They occurred in number of 1 to 26 specimens in the canalis vertebralis in one animal; the larvae were 4 — 7 mm. in length and 2 — 3.5 mm. thick. Following this period, the larvae occurred only under the skin either as encysted or (scarce in number) as loose ones.

Besides, 10 loose larvae, prior to boring the breathing hole in the skin, were utilized for the present study (7 — 10 mm. in length and approximately 4 mm. thick) and 50 larvae from the subcutaneous cysts. The latter two kinds of larvae were collected during the period from April 22 to June inclusive. The larvae from the subcutaneous cysts were 10 — 16 mm. in length and 6 — 10 mm. thick; they were whitish, and were beginning to darken.

The migratory larvae from the canalis vertebralis were extracted sterilely while the stationary ones were taken out unsterilely as the present author's auxiliary study had shown that the contents of the cysts following the drilling of opening by the larvae were not sterile on some occasions. Such larvae were sterilized by cooking prior to utilization.

Twenty species of Gram-positive and Gram-negative bacteria were utilized for testing the sensitiveness of the bacteria.

These species are listed below.

a) Gram-positive: 1. *Sarcina lutea,* 2. *Lactobacillus acidophilus,* 3. *Lactobacillus casei,* 4. *Staphylococcus albus,* 5. *Staphylococcus aureus,* 6. *Staphylococcus citreus,* 7. *Micrococcus lysodeicticus,* 8. *Erysipelothrix rhusiopathiae,* 9. *Streptococcus faecalis,* 10. *B. megatherium,* 11. *B. subtilis,* 12. *B. mesentericus.*

b) Gram-negative: 13. *Alcaligenes faecalis,* 14. *Salmonella typhosa,* 15. *Escherichia coli,* 16. *Proteus vulgaris,* 17. *Pseudomonas aeruginosa,* 18. *Salmonella typhimurium,* 19. *Serratia marcescens,* 20. *Pasteurella aviseptica.*

The above bacterial tribes were obtained from the Bacterial Tribe Bank of the Polish Academy of Sciences, Warszawa.

The body surface and intestinal sterility of the migratory larvae were tested by utilizing simple and selective media.

The antibacterial properties of the larvae were studied in Petri dishes with 2 per cent normal agar-agar, pH 7 — 7.4. The aseptically isolated intestines of the larvae were put on the dishes sown with the appropriate bacteria. The Hajna et Perry 1943 medium was utilized for sowing *Streptococcus faecalis* as it did not grown on the simple nutrient agar.

The smallest migratory larvae, in which the entire intestine was very difficult to isolate, were cut with a scalpel, the intestines taken out and triturated with a small amount of physiological solution in a mortar. The

cylinder method was applied. A part of the subcutaneous cysts were examined by the serial dilutions method using the centrifuged physiological solution trituration of these larvae. The dilutions were carried out in simple nutrient broth.

Experimental process and results

Sterility testing of the larvae: The sterilely extracted vertebral channel larvae were put into ordinary beef extract and kept for 24 to 48 hours at 37°C. Fourteen larvae thus examined proved sterile. Also those lying loosely under the skin, previously to the drilling an opening and encysting themselves, turned out sterile too. All sorts of the larvae utilized in the study were tested on their intestinal steriliaty. For this purpose the sterilely extracted intestine was triturated in a small amount of physiological solution, and then sown onto ordinary agar-agar media selective: to *Cocci* after Chapman 1951, to *Enterobacteriaceae* after Endo et Mc Conkey, and to *Streptococci* after Hajnaet Perry 1943. All of the thus examined intestines of several larvae of each sort showed to be sterile too.

Examination of bacteriostatic properties: The isolated intestine was put onto agar-agar dishes previously sown with appriopriate bacteria suspension. A 1:100 physiological solution of a 24 hour bacteria culture was utilized for sowing. The vertebral channel migratory larvae formed inhibition zones on no occasion, not even on the *Sarcina* recognized by Jettmar as the most sensitive ones. Inhibition zone did neither occur around the cylinder inserted into the agar-agar with *Sarcina lutea* and filled with the larval intestine trituration.

The above experiments with migratory larvae were repeated once more utilizing the bacteria: *Micrococcus lysodeicticus, Staphylococcus albus, Staph. aureus* and *Staph. citreus* as well as with the Gram-negative *E. coli* and *Salmonella typhimurium*. No inhibition of growth of these bacteria could be ever confirmed. As controls were used *Sarcina lutea* and *Micrococcus lysodeicticus* suspension sown dishes with inserted glass cylinders into which fresh body cavity liquid from *Parascaris equorum* was instilled. Now, the inhibition zone appeared.

In larvae laying loosely under the skin, and examined with utilization of the same technique as described above, the inhibition zone occurred first in conditions of an agar-agar culture of *Sarcina lutea*. Consequently, the assortment of bacteria examined was increased to cover the twenty bacterial tribes available. The results will be discussed in detail later on, when the next group of subcutaneous cyst larvae will be discussed, because they were identical.

The subcutaneous cyst larvae were examined by the method of putting down onto the dishes, and by that of successive dilutions of the centrifuged clarified liquid obtained by trituration of the larvae intestine in physiological solution.

Growth inhibition was obtained with the following Gram-positive bacteria: *Sarcina lutea, Micrococcus lysodeicticus, Staphylococcus albus, Staphylo-*

coccus aureus, Staphylococcus citreus, Erysipelothrix rhusiopathiae, Streptococcus faecalis. All of the above mentioned bacteria were also inhibited by larvae laying loosely under the skin, the inhibition zones being equally large in both groups.

The comparison of the bacterial inhibition results are given in the Table.

The different results obtained by the two authors in *Sarcina* and *Staphylococci* inhibition may be explaned by different properties of the cattle grub larvae utilized by the two authors or by different sensitiveness of the bacterial tribes utilized.

Differences in behaviour of these bacterial colonies which grew up within inhibition zones were confirmed in both of the above mentioned studies. Jettmar observed dwarf colonies which were significantly smaller than those growing outside the inhibition zone whereas, almost on all occasions, they were larger in my own experiments. A characteristic bank of colonies formed around the inhibition zone in both of the studies mentioned (see photographs).

Bacteria	Inhibition zone measured both ways in mm.	
	Jettmar 1953	Przyjałkowski
Sarcina	12	30
Staphylococci	3 - 12	10 - 15
Micrococcus lysodeicticus	-	28
Streptococcus scarlatinae	5	-
Streptococcus faecalis	-	6
Corynebacterium diphteriae	5	-
Gram-plus Coccobacillus from guinea pig	insignificantly	-
Bac. anthracis	insignificantly	-
Erysipelothrix rhusiopathiae	-	12

No inhibition occurred in the remaining Gram-positive bacteria utilized in the present study, namely: *B. megatherium, B. mesentericus, B. subtilis, Lactobacillus acidophilus* and *Lactobacillus casei.* The same was true of all of the Gram-negative bacteria utilized in the present study, namely: *Eberthella typhosa, Escherichia coli, Alcaligenes faecalis, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium, Serriatia marcescens* and *Pasteurella aviseptica.*

Tests with body cavity liquid and with other viscera such as the fat corpuscles, tracheae as well as with fragments of the chitin coat on no occasion led to any zone of bacteria growth inhibition in the Petri dishes when the latter were laid onto bacterial cultures which were sown previously.

Bacteriostatic properties were examined also by the cylinder method and by that of serial dilution method of the intestine contents in simple nutrient broth in test-tubes. This corroborated the qualitative results obtained by laying down the intestine into the bacteria sown Petri dishes.

The substance found in the intestine of *Hypoderma bovis* larvae laying loosely under the cow skin (previous to the drilling an opening in the skin by the larva), as well as that found in the intestine of the second stage larvae (from the subcutaneous cysts) seems to be analoguous to the substance confirmed by J e t t m a r in the third stage larvae.

The substance was thermostabile, dissolved both in physiological solution and in simple nutrient broth, diffused well even in 3 per cent. agar-agar. It resisted boiling water temperature for as long as up to 10 minutes.

The following are the results of experiments in which series dilutions were being applied utilizing clarified liquid after centrifuging the intestine triturate of *Hypoderma bovis* larva: full inhibition of growth of *Micrococcus lysodeicticus* in suspension occurred at dilution 1:50; partial inhibition — at 1:100 — 1:200. All of the cultures were incubated at temperature of 37°C. The results were checked after 24, 48 and 72 hours.

Growth inhibition occurred as early as the second day after sowing.

When the effect of the advisceral liquid from the *Ascaris suum,* and that from the *Parascaris equorum* on the one hand was compared to the bacteriostatic effect of the intestines of the *Hypoderma bovis* larvae on the other, higher effectivness of the latter was confirmed.

The relative investigations were carried out on Petri dishes applying the cylinder methods. Three cylinders were inserted into a Petri dish sown with bacteria responding to the effect of both the helminth advisceral liquid and of *Hypoderma bovis* larvae; the following was separately instilled into the cylinders: 1) *Ascaris suum* advisceral liquid, 2) *Parascaris equorum* advisceral liquid, and 3) triturate of *Hypoderma bovis* larvae from subcutaneous cyst. The widest inhibition zones were always obtained with the *Hypoderma bovis* larvae.

C o n c l u s i o n s

No bacteriostatic properties could be confirmed in the migratory *Hypoderma bovis* larvae from the vertebral channel by the methods applied. These properties appeared no earlier than after the migratory larvae had arrived under the skin and became stationary.

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STRESZCZENIE

Autor zbadał właściwości bakteriostatyczne larw *Hypoderma bovis*, stwierdzając, że opisywane przez Jettmar'a substancje bakteriostatyczne nie występują u larw pochodzących z kanału kręgowego bydła, a pojawiają się dopiero u larw „stacjonarnych", leżących luźno pod skórą bądź otorbionych. Te starsze larwy hamowały rozwój niektórych bakterii Gram-dodatnich, nie wpływając na bakterie Gram-ujemne. Autor przeprowadza porównanie z właściwościami wędrujących larw i dojrzałych postaci nicieni pasożytniczych.

EXPLANATION OF PLATE I

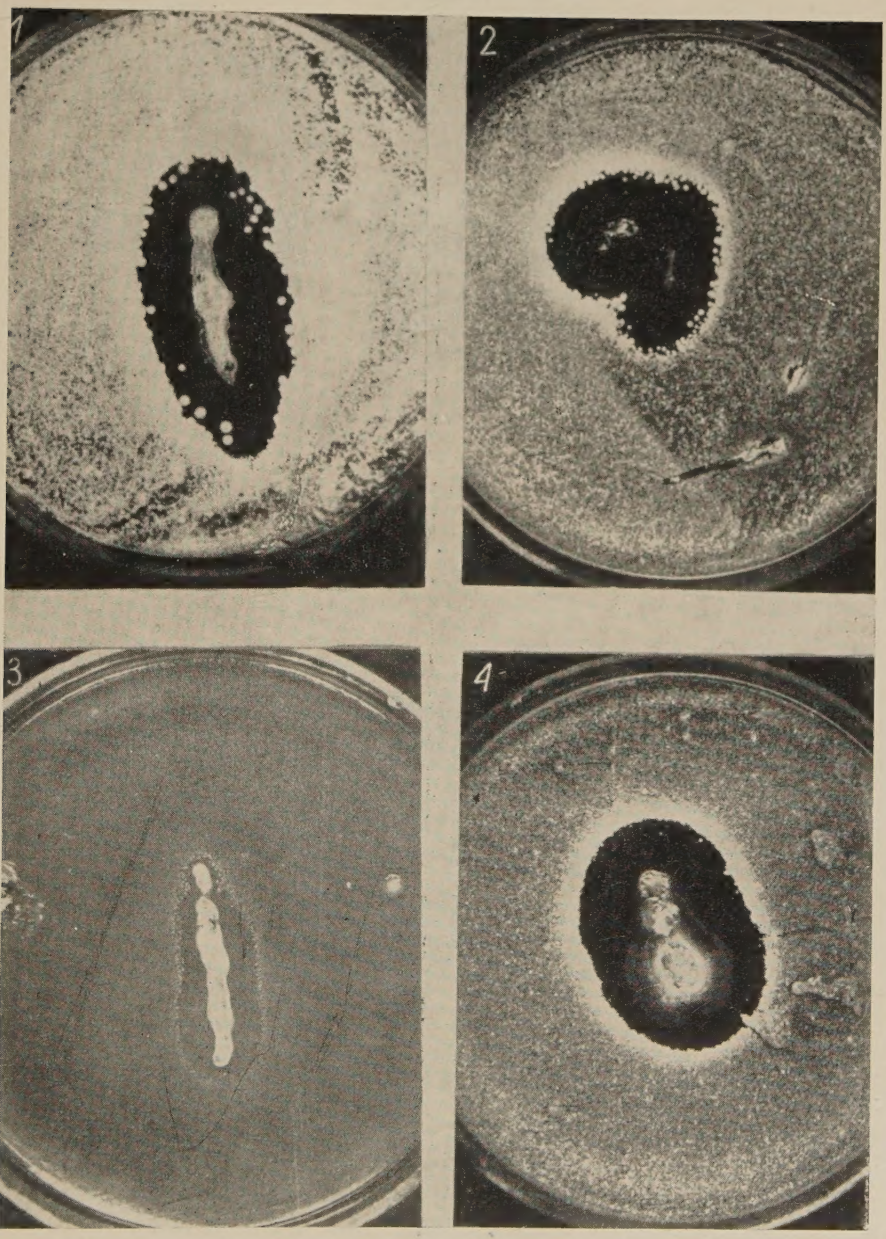
Phot. 1. Petri dish sown with *Sarcina lutea* (72 hours incubation at 37°C) with the intestine of the *Hypoderma bovis* larva at 2nd developmental stage (after cooking for three minutes). Note the wide wall of colonies around the inhibition zone and the separated colonies within the inhibition zone.

Phot. 2. Petri dish sown with *Micrococcus lysodeicticus* (72 hours incubation at 37°C) with pieces of the intestine of the 2nd stage larva extracted from the subcutaneous tissue prior to boring the opening in the skin. Next to the inhibition zone, other organs of the larva have been laid out.

Phot. 3. Petri dish sown with *Erysipelothrix rhusiopathiae*. In the centre: the intestine of a 2nd stage larva from a subcutaneous cyst.

Phot. 4. Petri dish sown with *Micrococcus lysodeicticus* (72 hours incubation at 37°C). In the centre: the intestine of a 2nd stage larva from a subcutaneous cyst (non sterilized). A distinct wall of colonies may be seen around the inhibition zone.

All of the photographs are 4/5 of natural size



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